

*Original Investigation*

In Vitro Effects of Mesenchymal Stem Cells and Various Agents on Apoptosis of Glioblastoma Cells

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AIM: To investigate a new anti-tumor treatment method using stem cells transfected with specific genes and proteins that induce apoptosis in tumor cells.

MATERIAL and METHODS: We used glioblastoma (GBM) cells and human adipose tissue-derived mesenchymal stem cells (AD-MSCs) in this study. The AD-MSCs were transfected with the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). To overcome apoptosis resistance in tumor cells, we used suberoylanilide hydroxamic acid (SAHA) as the histone deacetylase inhibitor and embelin as the X-linked inhibitor of apoptosis protein (XIAP). In addition, we silenced the XIAP gene on GBM cells with the shXIAP plasmid. Following the determination of half-maximal effective concentration (EC50%) doses of SAHA and embelin, GBM cells were incubated with them for 24 hours. XIAP-silenced and XIAP-non-silenced GBM cells were cultured with TRAIL-non-transfected and TRAIL-transfected stem cells for 24 hours. Viability and cell cycle analysis of all groups were determined using annexin V/propidium iodide and cell cycle method via flow cytometry.

RESULTS: TRAIL-transfected AD-MSCs, XIAP silencing, embelin, and SAHA induced apoptosis in GBM cells and decreased their proliferation, whereas TRAIL-non-transfected AD-MSCs did not.

CONCLUSION: Engineered stem cell therapies and molecular studies show promise in developing combination therapies for effective treatment of GBM.

KEYWORDS: Embelin, Glioblastoma, Mesenchymal stem cell, SAHA, TRAIL

INTRODUCTION

Glioblastoma (GBM) is a World Health Organization (WHO) grade IV astrocytoma (9). It is the most common and most aggressive primary brain tumor, accounting for approximately 60%–75% of all astrocytomas and 12%–15% of all intracranial tumors (27). The current treatment protocol for GBM is maximal safe surgical resection, radiotherapy, and chemotherapy (48). Despite this aggressive medical strategy, GBMs are either refractory or resistant to treatment (44). Even if gross total resection is confirmed by magnetic resonance imaging, local GBM recurrences are common because of microscopic tumor infiltration along perivascular spaces and

white matter tracts (11). Recurrence has also been attributed to genetic heterogeneity and the complex molecular pathology of GBM (20,29). Unfortunately, most systemic chemotherapeutics developed to treat GBM are ineffective because of their inability to cross the blood–brain barrier at certain doses without exerting systemic toxicity (31). Therefore, the treatment of malignant gliomas is one of the most difficult challenges in neuro-oncology. Recently, inspiring studies have found that targeted therapy that acts on specific signaling pathways that underlie uncontrolled cellular growth and induce apoptosis might be an effective and practical option for GBM treatment (13). Studies have also found that stem cells have tumor-specific homing properties and that programming

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them with genetic engineering to carry and release anti-tumor proteins that induce apoptosis is effective in *in vitro* and *in vivo* models of GBM cells (2,15,18,32,37,39,40).

Apoptosis is a physiologic, programmed cell death process that helps regulate homeostasis and is triggered by the activation of intracellular death program (34). One of the well-known tumor-specific proteins that induce apoptosis is tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). It exerts apoptotic effects via death receptors 4 and 5 in a wide variety of glioma cell lines, including intracranial human GBM xenografts in mice (2,35,40). Previous studies have shown that on-site delivery of TRAIL via stem cells has more anti-tumoral effects than administration of TRAIL alone (17,35). However, GBM cells might display resistance to apoptosis by increasing the synthesis of survival proteins or decreasing the synthesis of pro-apoptotic ones (36,43). One of the well-known proteins responsible for TRAIL-induced apoptosis resistance is the X-linked inhibitor of apoptosis protein (XIAP). It has been found that XIAP concentration is high in cancer cells that exhibit resistance to TRAIL-induced apoptosis (1,24).

Embelin is a small herbal molecule derived from *Embelia ribes*. Interestingly, embelin, which exerts anti-tumoral effects for different types of tumors by inhibiting XIAP, has no inhibitory effect on XIAP in GBM cells. Its anti-tumoral effect in GBM cells is via inhibition of different anti-apoptotic pathways (30). Another way of inhibiting apoptosis in different types of human tumors, including GBM (45,46), is by inhibiting some tumor suppressor genes via deacetylation by histone deacetylase (45). Histone acetylation–deacetylation is an important regulator of gene expression in normal and cancer cells (47). Suberoylanilide hydroxamic acid (SAHA) is a histone deacetylase inhibitor and has been demonstrated to slow the proliferation of GBM cells (46).

Additionally, the presence of GBM stem cells, which are a highly tumorigenic subpopulation of cancer cells extremely resistant to chemotherapy and radiotherapy, is said to be responsible for apoptotic resistance in GBM (22,42). Therefore, investigations for developing novel therapeutic regimens to cure GBM should focus on apoptotic resistance in both GBM cells and GBM stem cells (13).

In this *in vitro* study, we identified that mesenchymal stem cells (MSCs) may be used as a “Trojan horse” to carry an “assassin team” (TRAIL) to the target (GBM cells). We also identified that a combination of embelin, SAHA, and XIAP silencing potentiates the pro-apoptotic effects of TRAIL.

■ MATERIAL and METHODS

This study was approved by the Marmara University Local Ethical Council of Experimental Research on December 2, 2013 (45.2013.mar).

Cell Culture

Human U251 GBM cells (purchased from ATCC with the catalog number CRL-1690) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 100 U/mL penicillin and 100 U/mL streptomycin in a humidified atmosphere of 5% CO₂ and 95% air at 37°C.

Cell Passaging

After GBM cells became confluent, covering approximately 70%–80% of the flask surface area, they were transferred to another flask. The growth medium was changed every 3 or 4 days on the basis of pH changes. The cells were frozen with 90% fetal bovine serum (FBS) and 10% dimethylsulfoxide.

Cell Counting

The GBM cells in the flask were washed out from the growth medium with sterile phosphate-buffered saline (PBS). Trypsin/ethylenediaminetetraacetic acid, preheated to 37°C, was added to the flask (0.5 mL for a 25 cm² flask area; 1.5 mL for a 75 cm² flask area). The flask was stored in an incubator for 4–5 min. The cells taken from the flask's surface were put in 15 mL tubes. Next, 5 mL of FBS growth medium was added to each tube, and the tubes were centrifuged at 1300 rpm for 5 min. The supernatant was taken out of each tube, and the pellets were suspended in the growth medium. A 10 µL cell suspension was mixed with 10 µL trypan blue, and 10 µL of the suspension was put in a Thoma counting chamber. The cells were counted under a phase-contrast light microscope. The blue-stained cells were considered dead, whereas cells not stained with trypan blue were considered alive. The cell count in 1 mL of one cell suspension was calculated using the following formula:

$$\text{Cell count/mL} = \text{Mean count value (no. of cells in 64 squares/4)} \times \text{Dilution factor} \times 10^4$$

The cells were also counted using a Casy cell counter. A 10 µL suspended GBM cell sample was mixed with 10 mL Casy tone liquid and analyzed. The count of cells that were dead and alive was noted.

Determining the Inhibitory Concentration (IC₅₀) of Embelin and SAHA

The cytotoxicity of embelin and SAHA on GBM cells was determined by the WST-1 cell proliferation test. GBM cells were seeded in a 96-well plate in triplicate fashion. There were 5×10³ cells in each well. The cells were incubated for 24 hours to let them adhere to the wells. Predetermined amounts of embelin and SAHA were added to the wells separately. After 24 hours, 10 µL WST-1 solution was added to the wells. After a 4-hour incubation period, absorbance measurement was performed via a microplate absorbance reader.

Plasmids and Reagents

A plasmid vector expressing a fusion protein consisting of a green fluorescent protein (GFP) and the full length of human TRAIL (pEGFP-TRAIL) was bought from Addgene (plasmid #10953)(17). This plasmid contains kanamycin as a bacterial resistance gene and neomycin as an identification marker. The bacterial culture was incubated overnight at 37°C in Luria broth (LB) growth medium, which also contained 100 µg/mL kanamycin. On the next day, plasmid isolation was performed with a High Pure Plasmid Isolation Kit (Roche catalog no. 11754777001). The isolated plasmid was confirmed by a double-cut procedure with Bam I and EcoRI (Fermentas) restriction enzymes.

We used the shXIAP plasmid (Qiagen, SureSilencing, catalog no. 336311 KH00323G) for XIAP silencing. Inside the kit were four different XIAP sites and one negative control, and this plasmid had an ampicillin-resistant gene. The plasmid was transferred into *Escherichia coli* (*E. coli*) via transformation. For transformation, we first prepared competent cells via the CaCl_2 method. One 0.5 mL *E. coli* culture was added to 50 mL LB growth medium and centrifuged at 37°C at 180 rpm until the optical density of *E. coli* reached 0.6 (4.8×10^8 cells/mL). The bacterial culture was put into an ice solution for 10 minutes and then centrifuged at 4°C at 14,000 rpm for 4 minutes. Then, 10 mL CaCl_2 was added over the pellet and left in ice for 30 minutes. The solution was re-centrifuged at 4°C at 14,000 rpm for 4 minutes. The pellet was suspended in 2 mL 0.1 M CaCl_2 solution and stored at 4°C. For transformation; we took one vial of competent *E. coli* (BL-21 strain), which was stored at -80°C and was held in ice for 15 minutes. Then, half of the vial (100 μL) was put in a 1.5 mL tube, and the remaining half was stored as the control group. Two 2 μL shXIAP plasmids were mixed with *E. coli* in the tube, which was placed on ice for 30 minutes, then kept at 42°C for 90 seconds, and again placed on ice for 5 minutes. Next, 900 μL LB growth medium was added and shaken via a vortex at 300 rpm at 37°C. After 80-minute incubation at 37°C, the mixture was centrifuged at 4500 rpm for 10 minutes. The pellet was mixed with 100 μL LB growth medium and cultivated on an ampicillin-containing LB petri dish. The petri dish was incubated at 37°C overnight. After incubation, one colony was chosen from the petri dish and cultivated in LB growth medium containing 100 $\mu\text{g}/\text{mL}$ ampicillin. The next day, plasmid isolation was performed with a High Pure Plasmid Isolation Kit (Roche catalog no. 11754777001). The entire procedure was repeated for four different XIAP regions.

PstI enzyme (Fermentas catalog no. ER0611) was used for plasmid confirmation because the plasmid had a PstI enzyme cleavage site. For this procedure, 5 μL plasmid DNA (mass was 1 μg or more), 2 μL 10X Buffer O, 1 μL PstI cleavage enzyme, 10 μL DNase, and RNase-free water were mixed in a tube. The mixture was incubated at 37°C for 45 minutes and then monitored with 1% agarose gel electrophoresis at 100 V for 40 minutes.

Transfection of pEGFP-TRAIL Vector into Adipose Tissue-Derived MSCs and Fibroblasts with Electroporation

Transfection of the pEGFP-TRAIL plasmid vector to human adipose tissue-derived MSCs (AD-MSCs) and fibroblasts was performed with an Amaxa Nucleofector machine. Cells were grown in a culture plate, trypsinized, and stored. We prepared 1×10^5 cells for each well and put them in 100 μL nucleofector solutions. Then, 1.5 μg of plasmids was added, and all mixtures were transferred to cuvettes. Cuvettes were placed in the nucleofector machine and transfected via the FF-104 program. After transfection, a growth medium was added over the cuvettes and cells were put in 24-well plates.

Transfection of the shXIAP Plasmid into GBM Cells

For each well, 5×10^5 GBM cells were placed in 24-well plates, which contained DMEM. Transfection was done after the GBM

cells covered 60%–70% of the surface. Lipofectamine 2000 (Invitrogen) transfection solution was used for transfection. The procedure was performed by using 24-well plate ratios as per manufacturer's instructions. Plasmid DNA (0.8 μg) was diluted in 50 μL serum-free DMEM, and 2 μL transfection solution was diluted in 50 μL serum-free DMEM and kept at room temperature for 5 minutes. Both mixtures were combined together and kept at room temperature for 20 minutes. Then, the 100 μL mixture was slowly added to the wells. The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Every 4–6 hour, the growth medium of the cells was replaced, and after a 24-hour incubation period, transfection of shXIAP was confirmed under a fluorescence microscope.

Choosing the Most Effective XIAP-Silencing Region in GBM Cells

The shXIAP plasmid (Qiagen) had four different regions that act on different sites of the target gene for silencing. These regions were applied to the cells one by one. The region that was most effective for XIAP silencing was chosen for ongoing experiments. Gene expressions were tested via real-time polymerase chain reaction (RT-PCR). After transfection into the cells, RNAs were isolated from the cells via the RNeasy Mini RNA isolation kit (Qiagen, catalog no. 74104). Complementary DNAs (cDNAs) were synthesized from RNAs by using the Strand cDNA Synthesis Kit (Fermentas, catalog no. K1652).

Co-culture of Human AD-MSCs and GBM Cells

GBM cells with an active gene and an inactive XIAP gene were put in 6-well plates with DMEM. Each well contained 1×10^6 cells. The cells were incubated in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. After incubation for 24 hours, the GBM cells were put into 0.4-inch-diameter pore-sized cell culture inserts (BD-353090). TRAIL-transfected and TRAIL-nontransfected human AD-MSCs and fibroblasts were added to the inserts. The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Our experimental groups were as follows:

- Group 1 (Control group): GBM cells and fibroblasts were co-cultured.
- Group 2: GBM cells and human AD-MSCs were co-cultured.
- Group 3: GBM cells and TRAIL-transfected human AD-MSCs were co-cultured.
- Group 4: GBM cells and TRAIL-transfected human AD-MSCs were co-cultured, and embelin was added to the medium.
- Group 5: GBM cells and TRAIL-transfected human AD-MSCs were co-cultured, and SAHA was added to the medium.
- Group 6: XIAP-silenced GBM cells and TRAIL-transfected human AD-MSCs were co-cultured, and embelin and SAHA were added to the medium.

Annexin V/PI Assay

Next, 2 mL of 5×10^5 cells were put in 6-well plates and incubated at 37°C for 24 hours in a humidified atmosphere of 5% CO₂ and 95% air. The experimental groups were organized and incubated as previously described. The content of each well was poured into a different tube and used for the annexin V/propidium iodide (PI) apoptosis assay and cell cycle regulation analysis. The cell suspensions for the annexin V/PI assay were centrifuged at 1300 rpm for 5 minutes. Each pellet was suspended with 115 µL of a fluorescent reactive. The tubes were incubated in the dark for 15 minutes, and then 400 µL PBS was added to each tube. The cell suspension in each tube was analyzed by a flow cytometer with FL1 and FL2 filters. The results were used for statistical analysis.

Statistical Analysis

The outputs were analyzed with one-way analysis of variance in GP Prism5—GraphPad Software (San Diego, CA, USA, 2007) and reported as a mean standard deviation.

RESULTS

IC₅₀ of Embelin and SAHA

The IC₅₀ values of embelin and SAHA on human U251 GBM cells were found to be 75 and 3 µM, respectively (Figures 1, 2). The embelin and SAHA fraction-affected values were calculated using the CalcuSyn software as 50 and 1.5 µM, respectively.

Confirmation of shXIAP Transfection under a Fluorescence Microscope and Determination of the Most Effective XIAP-Silencing Region on GBM Cells

Under a fluorescence microscope, the GFP confirmed the transfection of the shXIAP plasmid into GBM cells (Figure 3). The most effective XIAP-silencing region on GBM cells was determined with RT-PCR. The most effective gene silencing was observed on the C2 region (Figure 4). With these findings, we continued our experiments with XIAP clone 2.

Determination of Apoptosis with Annexin V/PI Assay via Flow Cytometry

The annexin V/PI assay was performed for all experimental groups. For each step, 10^5 cells were counted. The GBM cells that were alive were annexin V negative and PI negative; the early apoptotic GBM cells were annexin V positive and PI negative; the late apoptotic GBM cells were annexin V positive and PI positive; and the necrotic GBM cells were annexin V negative and PI positive. The percentage of GBM cells in each category (alive, early apoptotic, late apoptotic, and necrotic) was calculated for all experimental groups. The sum of the percentages of early and late apoptotic GBM cells in each experimental group was accepted as the percentage of the total apoptotic GBM cells for that experimental group. The percentage of apoptosis in each group is depicted in a bar chart in Figure 5.

Compared with the control group, no statistically significant increase in apoptosis was observed in the GBM/AD-MSC

group ($p > 0.05$). There was a statistically significant increase in apoptosis in the GBM/TRAIL+AD-MSC group, the GBM/TRAIL+AD-MSC+embelin group, the GBM/TRAIL+AD-MSC+SAHA group, and the XIAP-GBM/TRAIL+AD-MSC+embelin+SAHA group in comparison with the control group ($p < 0.05$). The most effective apoptosis was observed in

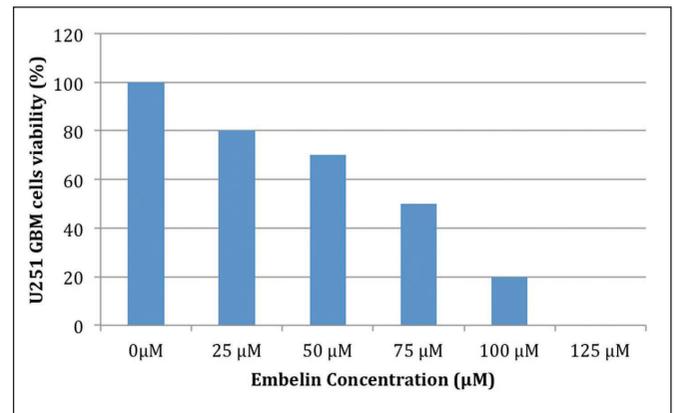


Figure 1: EC₅₀ value of embelin.

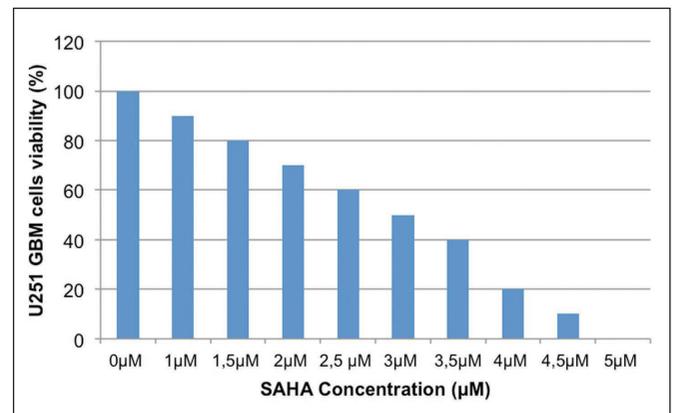


Figure 2: EC₅₀ value of SAHA.

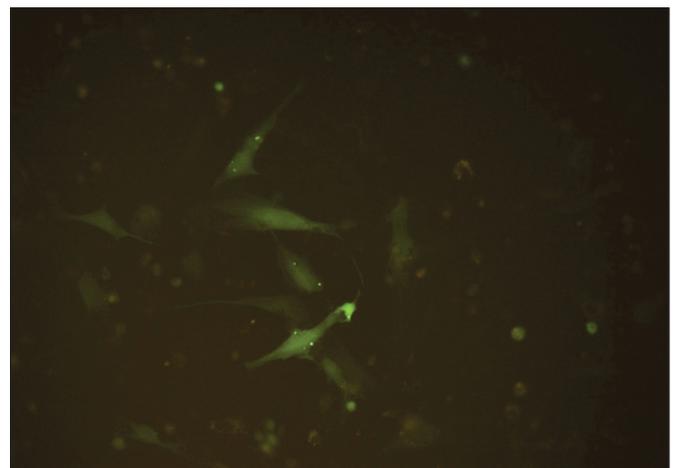


Figure 3: Green fluorescence illumination under a fluorescence microscope confirms the shXIAP plasmid transfection of GBM cells.

the GBM/TRAIL+AD-MSC+embelin+SAHA+XIAP-silencing group, and the apoptosis rate was significantly higher than that of any other experimental group in the study ($p < 0.05$).

DISCUSSION

This study was performed to evaluate the effects of MSCs, TRAIL, XIAP silencing, embelin, and SAHA on the apoptosis of human U251 GBM cells.

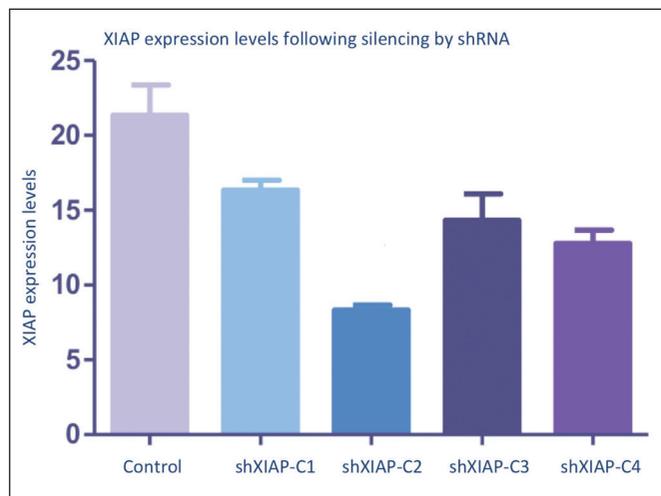


Figure 4: The most effective XIAP-silencing region on GBM cells was determined with RT-PCR. The most effective gene silencing was observed on the C2 region.

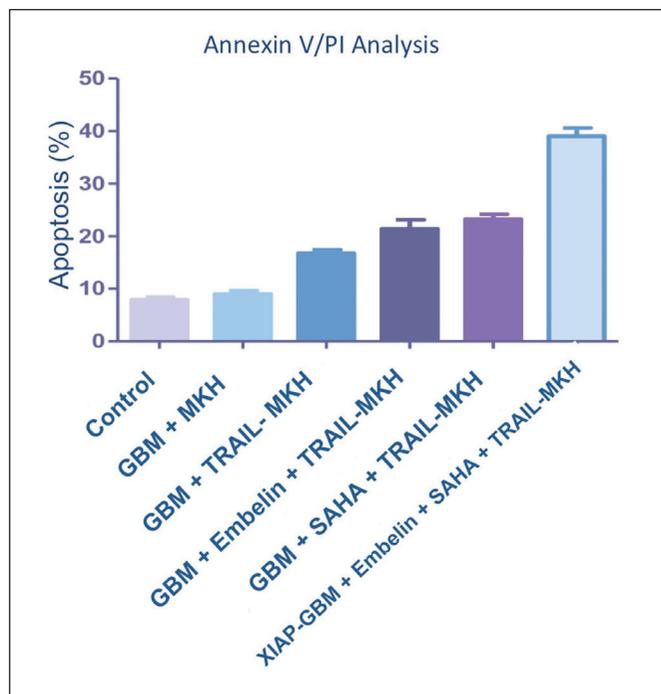


Figure 5: Bar chart showing the percentage of apoptosis in each experimental group. The highest apoptosis was observed in the GBM/TRAIL-AD-MSC+embelin+SAHA+XIAP-silencing group.

A variety of cancer-related molecular alterations have been identified in recent years. Some of these alterations are supposed to be potential targets for GBM treatment. One of the new and promising experimental treatment strategies for GBM is the re-activation of TRAIL-induced apoptosis. TRAIL is responsible for normal homeostasis, immune surveillance, determination of self/non-self-cells, immunomodulation, and elimination of virally infected cells and cancer cells (14,16,21).

TRAIL-induced apoptosis seems to be an excellent anticancer treatment strategy for GBM because TRAIL selectively induces apoptosis in tumor cells, sparing normal cells (6,28,33). Recent studies have shown that sustained levels of TRAIL are needed for effective TRAIL-induced apoptosis in cancer treatment (18). Most studies investigating TRAIL-induced apoptosis for cancer treatment have used human or mouse MSCs for sustained synthesis and release of TRAIL to the tumor microenvironment (3,8,10,18,23,37,38). In this study, we used human AD-MSCs for TRAIL synthesis and secretion.

TRAIL is an effective antitumor agent, but nearly more than 50% of GBM lines are resistant to TRAIL-induced apoptosis (18,28,33). Therefore, identification of survival signals that help GBM cells escape from TRAIL-induced apoptosis is crucial for designing TRAIL-associated combination regimens for GBM treatment. Overexpression of proteins that inhibit apoptosis is one of the most blamed mechanisms that induce TRAIL resistance in GBM cells. Most of these proteins belong to a family called the inhibitor of apoptosis protein (IAP) family. Griffith et al. and Ng et al. reported that two members of the IAP family, survivin and XIAP, are responsible for TRAIL-induced apoptosis resistance in certain cancer cells (12,25). These two proteins inhibit apoptosis by interfering with the activities of caspases 3 and 7 (7). Both proteins are overexpressed in GBMs, and their high expressions correlate with a decreased survival rate, increased recurrence, and increased resistance to therapy (4,19). In this study, we blocked XIAP synthesis by shXIAP-silencing of the XIAP gene in GBM cells. This led to a dramatic increase in the apoptosis of GBM cells in comparison with XIAP-expressing groups.

During discussions of XIAP silencing to overcome resistance to TRAIL-induced apoptosis, a new XIAP inhibitor molecule called embelin was discovered. Embelin is extracted from the fruit of *E. ribes*, and has anti-inflammatory, analgesic, and anti-tumor properties. It induces apoptosis in different types of human cancer cells by inhibiting NF- κ B, STAT3, peroxisome proliferator-activated receptor gamma, and XIAP (5,26,30). Siegelin et al. showed that embelin enhanced TRAIL-induced apoptosis in human U251 and LN229 glioma cells by reducing the expression of cellular FLICE-like inhibitory protein (41). Park et al. showed that embelin exerts pro-apoptotic effects in GBM cells by inhibiting the NF- κ B pathway, not the XIAP pathway (30). In this study, addition of embelin into TRAIL-transfected AD-MSCs and GBM co-culture media resulted in a statistically significant increase in the apoptosis of GBM cells.

We also tested the effects of SAHA, a histone deacetylase inhibitor, on the apoptosis of GBM cells. Yin et al. showed that SAHA inhibited the growth of GBM cells, with accumulation

in the G2-M checkpoint. SAHA equally inhibited the growth of both p53 mutant and p53 wild-type glioma cells (46). This result showed that in GBM cells, the anti-proliferative effect of SAHA is independent of p53 activity. In our study, addition of SAHA into TRAIL-transfected AD-MSCs and GBM co-culture media resulted in a statistically significant increase in the apoptosis of GBM cells.

In this *in vitro* study, we also found that AD-MSCs by themselves have no statistically significant effect on the apoptosis of GBM cells. But the production and secretion of TRAIL by genetically engineered, TRAIL-plasmid-transfected AD-MSCs resulted in a dramatic increase in the apoptosis of GBM cells. Addition of embelin to one co-culture medium of TRAIL/AD-MSCs and addition of SAHA to another co-culture medium of TRAIL/AD-MSCs resulted in increased apoptosis of GBM cells. However, there was no significant difference in apoptosis between both groups. Finally, we saw a second dramatic increase in apoptosis in a combination of all groups including XIAP silencing.

■ CONCLUSION

Our study demonstrated the effects of TRAIL-transfected AD-MSCs, embelin, SAHA, and XIAP silencing on the apoptosis of GBM cells. We observed the maximum apoptosis in the GBM/TRAIL/AD-MSCs+embelin+SAHA+XIAP-silencing group.

These results are promising for future combination therapies for GBM. Using this study as a template, new *in vivo* studies can be planned and novel engineered stem cell transplantation therapies in combination with other anti-cancer agents can be developed for GBM patients.

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